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13. ABSTRACT (Maximum 200 Words)

The inability to inhibit the activity of CDK4/6 kinase is implicated in a significant number of breast cancers, making it an ideal molecule for targeted inhibition. We propose to prepare CDK4/6-specific inhibitors that are based on the naturally occurring CDK4/6 specific inhibitory protein. $p18^{INK4c}$. Towards this goal, we have used structure-based-design to successfully prepare three mutant $p18^{INK4C}$ proteins, F71N, F82Q and F92N, with increased stability. Significantly, the F71N mutant also shows enhanced CDK6 interaction and cell cycle inhibitory activity *in vivo*. We are currently preparing other mutations at positions 71, 82 and 92 of $p18^{INK4c}$ to generate even more potent CDK4/6 inhibitors. Once this analysis is complete we will combine favorable mutants to create a "super stable" $p18^{INK4c}$ protein that will be used as a scaffold to prepare small peptides derived from the optimally modified $p18^{INK4c}$ protein. The preparation of this initial peptide will than lead to a set of lead peptides or non-peptidic compounds that can be further developed using combinatorial chemistry approaches. High affinity compounds developed through this approach can then be tested in cell culture systems and ultimately through clinical trials to treat CDK4/6-mediated breast cancers.

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Table of Contents

(5) INTRODUCTION

Distinct cancer types have been correlated with several proteins that are involved in the G1 to S transition of the mammalian cell cycle (Funk, 1999; Molinari, 2000). In particular, the inability to inhibit the activity of the paralogs cyclin-dependent kinases 4 and 6 (CDK4/6) are implicated in more than 80% of human neoplasias (Ortega et al., 2002). For example, the gene encoding the CDK4/6 inhibitory protein, $p16^{INK4}$, is deleted or mutated in the majority of leukemias, bladder cancers and familial melanomas (Roussel, 1999). The CDK4/6 stimulatory subunit, cyclin D1, is commonly found to be overexpressed or gene amplified in spontaneous breast cancers (Donnellan and Chetty, 1998; Khoo et al., 2002), and overexpression of cyclin D1 in mice leads to death due to breast cancer (Wang et al., 1994). Finally, CDK4 itself is overexpressed or gene amplified in about one third of breast cancers (Ortega et al., 2002). Together, these observations indicate that deregulation of the G1 to S transition of the mammalian cell cycle is tightly linked to the onset of several different cancer types, and that the CDK4/6 protein, in particular, is an excellent candidate for targeted inhibition for the treatment of breast cancer.

The vast majority of currently available CDK4/6 inhibitors have structural similarity to the common kinase cofactor ATP and, as a result of this, a major obstacle in developing these inhibitors into clinically useful drugs is overcoming harmful side effects due to a lack of CDK4/6 specificity (Toogood, 2001). Here, we propose to prepare a CDK4/6-specific kinase inhibitor that is based on the naturally occurring CDK4/6 specific inhibitory proteins of the INK4 family (Carnero and Hannon, 1998). We have previously determined the structure of the INK4 protein $p18^{INK4c}$ revealing 5 contiguous ankyrin-like repeats (Venkataramani et al., 1998), and the structure of CDK6 in complex with $p18^{INK4c}$ (Jeffrey et al., 2000) reveals that repeats 2 and 3 mediate most of the inhibitory interactions with CDK6. Based on these results, we hypothesize that a modified and truncated $p18^{INK4c}$ protein (INK4-mod_{2-3}) can be prepared harboring only ankyrin-like repeats 2 and 3 in addition to amino acid substitutions that enhance the proteins CDK4/6 inhibitory activity. Modifications in $p18^{INK4c}$ will include amino acid substitutions that increase the stability of the inhibitory domain in order to raise the likelihood of autonomous folding, and to increase the affinity of $p18^{INK4c}$ for CDK4/6. INK4-mod_{2-3} will then serve as a potent CDK4/6-specific peptide inhibitor itself as well as an excellent scaffold for the design of even smaller peptide or small molecule non-peptidic mimics that may have clinical application for the specific inhibition of CDK4/6 for the treatment of breast cancer.

The Specific Aims of the proposal are to (1) Prepare and characterize site-directed mutants of $p18^{INK4c}$ with increased protein thermostability, association with CDK4/6, and cell-cycle inhibitory activity *in vivo*, (2) Prepare an autonomous ankyrin-like region 2-3 peptide derived from the optimally modified $p18^{INK4c}$ (INK4-mod_{2-3}) protein prepared in aim 1, (3) Determine the X-ray crystal structure of INK4-mod_{2-3} in complex with CDK6, and (4) Use the structural information from the CDK6/ INK4-mod_{2-3} complex to initiate the structure-based design of peptide or small molecule non-peptidic mimics of INK4-mod_{2-3}.

(6) BODY

During the first year of the funding period we completed most of Aim1 (Tasks 1-3) to prepare and characterize site directed mutants of $p18^{INK4c}$ with increased protein thermostability, association with CDK4/6 and cell-cycle inhibitory activity *in vivo* (Venkataramani et al., 2002). Specifically, we used a combination of structure-based mutagenesis, structural studies, and *in vitro* and *in vivo* studies to identify three $p18^{INK4c}$ mutant proteins with increased protein thermostability and CDK4/6 inhibitor activity, F71N, F82Q and F92N. Of these three $p18^{INK4c}$ mutant proteins the F71N mutant showed the most significant enhancement in protein stability and CDK4/6 inhibitory activity.

An assumption of the studies with $p18^{INK4c}$ was that the mutations that stabilize this member of the INK4 proteins would also stabilize the $p16^{INK4a}$ protein, thereby allowing us to use $p18^{INK4c}$ as a suitable model system for designing small $p16^{INK4a}$ mimics for CDK4/6 inhibition. Before moving forward with our studies we tested our assumption by preparing $p16^{INK4a}$ mutations that correspond to the F71N, F82Q and F92N mutations prepared for $p18^{INK4c}$. Therefore, we prepared the $p16^{INK4a}$ mutant proteins T79Q, T79N, F90N and A100N. Each of these mutant proteins were prepared by site-directed mutagenesis, and the proteins overexpressed in bacteria as inclusion bodies. The proteins were then solubilized by suspending them in 6M urea and refolded them by dialysis against a buffer in the absence of urea. Each of the soluble and refolded proteins was chromatographed on gel filtration using a Superdex-75 column. A summary of the gel filtration chromatographs and SDS-PAGE analysis of the mutant $p16^{INK4a}$ proteins is summarized in figure 1 (appendix). Surprisingly, we found that while the majority of the native $p16^{INK4a}$ protein chromatographs as a monomeric protein, each of the mutants show a large percentage of protein in the aggregated form (in the void volume of the column), suggesting that each of the $p16^{INK4a}$ mutants that we prepared are less stable than the native protein. Crystallization trails with each of these $p16^{INK4a}$ mutant proteins were also unsuccessful. We conclude from these studies that $p18^{INK4c}$ may not serve as a suitable model for designing more thermostable $p16^{INK4a}$ proteins. In light of this, we have now modified our Aims to directly study site-directed mutations and protein truncations in $p16^{INK4a}$, rather than $p18^{INK4c}$.

While we were designing stabilizing mutations in $p16^{INK4a}$, we noted a manuscript published by Peng and colleagues in which the authors used several complementary techniques to identify three stabilizing mutations in $p16^{INK4a}$ (Cammett et al., 2003). These mutations are W15D, L37S and L121R. Interestingly a combination of these stabilizing mutations (called HTM for hyperstable triple mutant) has an additive thermostability with the HTM mutant being 1.4 kcal/mol more stable than the wild-type protein. In addition, the HTM $p16^{INK4a}$ mutant was able to rescue the oncogeneic R24P, P81L and V126D $p16^{INK4a}$ mutants for CDK4 binding. We have decided to take advantage of these seminal findings by preparing the HTM $p16^{INK4a}$ mutant protein for our studies.

Over the coming year we will (1) Prepare a recombinant form of the HTM $p16^{INK4a}$ mutant, and (2) purify and crystallize the HTM $p16^{INK4a}$ protein and determine its three dimensional structure. The HTM p16 structure will then serve as a structural scaffold for Aims 2 and 3 of this proposal.

(7) KEY RESEARCH ACCOMPLISHMENTS

- Prepared recombinant $p16^{INK4a}$ mutant proteins (T79Q, T79N, F90N and A100N) that were predicted to have increased thermal stability.
- Characterized the gel filtration behavior of the $p16^{INK4a}$ mutants described above leading to the conclusion that the INK4 protein $p18^{INK4c}$ does not serve as a good model for designing more thermostable $p16^{INK4a}$ proteins.

(8) REPORTABLE OUTCOMES

None

(9) CONCLUSIONS

During the first year of the funding period we have successfully completed tasks 1-3 of the proposal. Specifically, we have prepared $p18^{INK4c}$ mutant proteins that harbor increased protein stability and CDK4/6 inhibitory activity. During the second year of the funding period we addressed whether the INK4 protein, $p18^{INK4c}$, serves as a good model system for understanding the structure and stability of $p16^{INK4a}$ and discovered that it does not. In light of these findings and a recent report by Peng and colleagues describing the preparation of a more thermostable and active $p16^{INK4a}$ protein ($p16^{INK4a}$ -HTM), we have shifted our focus to $p16^{INK4a}$ -HTM for our subsequent studies (Aims 2 and 3).

Since the hyperactivity of the CDK4/6 kinase is associated with a large number of cancers including a significant number of breast cancers, CDK4/6 is a highly relevant target for the development of inhibitory compounds that may provide effective therapeutics for the treatment of breast cancer. Unfortunately, the vast majority of currently available CDK4/6 inhibitors have structural similarity to the common kinase cofactor ATP and, as a result of this; a major obstacle in developing these inhibitors into clinically useful drugs is overcoming harmful side effects due to a lack of CDK4/6 specificity. Our use of the INK4 family as a scaffold will result in the preparation of peptide inhibitors of CDK4/6 with enhanced specificity. The preparation of these initial peptides will than lead to a set of lead peptides or non-peptidic compounds that can be further developed using combinatorial chemistry approaches (Beeley and Berger, 2000; Kirkpatrick et al., 1999; Leach et al., 2000; Roe et al., 1998). High affinity compounds developed through this approach can then be tested in cell culture systems and ultimately through clinical trials to treat CDK4/6-mediated breast cancers.

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(11) APPENDIX

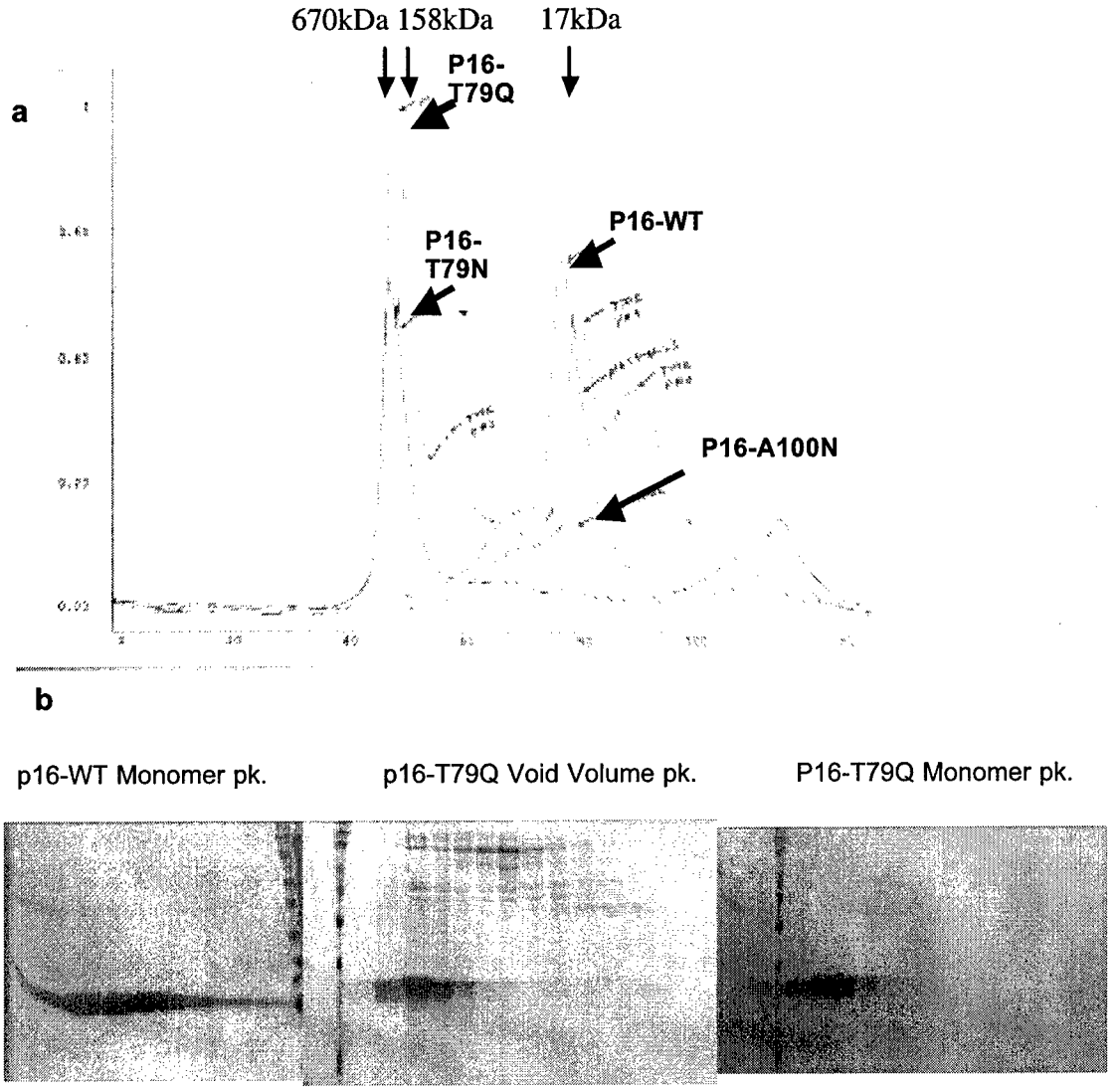

Figure 1. (a) Overlay of gel filtration chromatograph of wild-type $p16^{INK4}$ and several representative site-directed $p16^{INK4}$mutants (indicated on figure). (b) SDS-PAGE analysis of wild-type $p16^{INK4}$ and the T79Q $p16^{INK4}$ mutant.